PROPER CORONARY VASCULAR DEVELOPMENT AND HEART MORPHOGENESIS DEPEND ON INTERACTION OF GATA-4 WITH FOG COFACTORS

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PROPER CORONARY VASCULAR DEVELOPMENT AND HEART MORPHOGENESIS DEPEND ON INTERACTION OF GATA-4 WITH FOG COFACTORS.

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GATA-family transcription factors are critical to the development of diverse tissues. In particular, GATA-4 has been implicated in formation of the vertebrate heart. As the mouse *Gata-4* knock-out is early embryonic lethal because of a defect in ventral morphogenesis, the in vivo function of this factor in heart development remains unresolved. To search for a requirement for *Gata-4* in heart development, we created mice harboring a single amino acid replacement in GATA-4 that impairs its physical interaction with its presumptive cardiac cofactor FOG-2. *Gata-4* ki/ki mice die just after embryonic day (E)12.5 exhibiting features in common with *Fog-2* -/- embryos as well as additional semilunar cardiac valve defects and a double-outlet right ventricle. These findings establish an intrinsic requirement for GATA-4 in heart development. We also infer that GATA-4 function is dependent on interaction with FOG-2 and, very likely, an additional FOG protein for distinct aspects of heart formation.

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Introduction

The process of mammalian heart development involves the specification of many different cell lineages, including coronary vasculature cells, endocardial cells, and cardiomyocytes. These cells must assemble into the mature four-chambered heart in a specific, complex, and carefully structured manner. This crucial assembly occurs in early embryonic development in the mouse, beginning at embryonic day 7.5 and lasting through day E11. Viability of mammals requires the proper synthesis of a beating heart as well as a functioning fetal circulatory system. Therefore, it follows that severe defects in cardiac development result in embryonic lethality between days 8.5 and 12, while those less severe defects result in congenital heart disease(1).

The specific stages of cardiac development have been characterized. Murine cardiac embryogenesis involves three stages. During the first stage, beginning at day E 7, the procardiomyocyte cells differentiate from the cells of the splanchnic mesoderm. During the second stage, beginning at day E 8, these procardiomyocytes migrate from the dorsal and anterior regions of the embryo to form a linear heart tube. In the third stage of murine cardiac embryogenesis, occurring between days 8.5 and 11, septation and looping occur, transforming the linear heart tube into the familiar four chambered heart (1).

Recent advances in the field of molecular biology have identified transcription factors that are responsible for the regulation of each stage of cardiac development. Many researchers have characterized transcriptional regulatory elements that are expressed in the developing mammalian heart. One family of transcription factors that has emerged as potential regulators of cardiac development includes the GATA zinc finger family of proteins. Current research is directed at better understanding the exact role of the GATA transcription factors in the regulation of cardiac development.

Transcriptional activity of the GATA-factors is modulated through interaction with nuclear proteins, including zinc finger proteins of the Kruppel and FOG/U-shaped families, general coactivators (p300 and CBP), the myocardial-expressed protein Nkx2.5, and NF-

AT3 (2, 3, 4, 5).Whereas the specificity and in vivo functional relevance of many of these interactions are incompletely defined, the association of GATA-1 with FOG-1 has been examined in detail. FOG-1 interacts with GATA-1 in hematopoietic cells and regulates the ability of GATA-1 to promote terminal differentiation of erythroid cells and megakaryocytes (6). Mutation of specific residues within the conserved N-terminal zinc finger of GATA-1, such as V205G, disrupts binding to FOG-1, preserves DNA-binding properties of GATA-1, and renders GATA-1 unable to promote terminal differentiation of red blood cells (7). Furthermore, mutation of Val 205 in humans leads to congenital dyserythropoietic anemia and thrombocytopenia (8).Taken together, these findings demonstrate that direct physical association of GATA-1 and FOG-1 is essential for GATA-1 's roles in transcription and, critical for the experiments reported herein, identifies a specific residue of the N finger that mediates cofactor interaction.

GATA-4, GATA-5, and GATA-6, nonhematopoietic expressed factors, are implicated in development of heart, endoderm, and intestinal epithelia, where they are expressed in an overlapping and dynamic fashion (9, 10, 11, 12, 1, 4). GATA-4 has been extensively studied in the context of heart development, as it is present in precardiac splanchnic mesoderm and binds to and activates promoters and enhancers of numerous myocardial-expressed genes (14). In its absence, mouse embryos die by E7.0 –9.5, with failure of ventral morphogenesis leading to cardiac bifida (15, 16). The death of the *Gata4* null embryos before formation of a heart tube precludes analysis of the role of this factor in later cardiac organogenesis.

The second member of the FOG protein family, FOG-2, is expressed in cardiac and nervous system tissues and interacts with the N fingers of all GATA factors, including those expressed within the developing heart (GATA-4, GATA-5, and GATA-6; (17, 18, 19). As such, it is a candidate cofactor for these GATA factors in the heart. Mouse embryos lacking *Fog2* die of heart failure between E12.5 and E15.5 (20, 21). Fog- 2 -/- hearts exhibit a constellation of defects, such as overriding aorta, subpulmonic stenosis, and subaortic

ventricular septal defect (VSD), seen in the human congenital malformation Tetralogy of Fallot. In addition, despite formation of an intact epicardial layer and expression of epicardium-specific genes, initiation of coronary vasculature fails to take place. This is evidenced by the absence of induction of markers of coronary vessel development, including ICAM-2 and Flk-1 in *Fog2* -deficient hearts. These defects in coronary vasculature formation are secondary to lack of *Fog2* expression specifically in myocardium, as demonstrated by transgenic rescue (21).

Statement of purpose and hypothesis

The underlying goal of our research is to better understand the molecular regulation of mammalian cardiac development. Specifically, we hope to gain insight into the role of the interaction between two transcription factors, GATA-4 and FOG-2, in the developing heart. The GATA family of transcription factors play important roles in the development of multiple organs. In particular, GATA-4 is believed to participate in heart formation. Previous studies using knock-out gene targeting for GATA-4 has failed to confirm this, because the animals die at points in embryogenesis prior to heart maturation. In contrast, the putative GATA-4 cofactor FOG-2 (friend of GATA) is known to be absolutely required for heart formation, as mice with targeted deletion of this gene die between E12.5 and E15.5 due to severe heart failure (21). To determine whether FOG-2 functions through GATA-4, we created a mutant strain of mice that harbor a single point mutation in the GATA-4 gene. This mutation alters an amino acid that is required for the interaction between GATA-4 and FOG proteins, including FOG-2. Consequently, in our mutant "GATA-4 knock-in" animals, GATA-4 and FOG-2 can no longer bind one another. Through examining these mice, we attempted to determine the developmental events affected by disrupting the interaction between FOG proteins and GATA-4. We hypothesized that mice homozygous for this mutation would die in embryogeneis between E 12.5 and E15.5 of heart failure, and would exhibit a phenotype similar to that of the FOG-2 -/- embryos, thereby illustrating that FOG-2 acts exclusively through GATA-4. We hypothesized that novel cardiac defects would possibly be seen in the GATA-4 knock-in animas in addition to those present in the FOG-2 knock-out, providing evidence that GATA-4 functions through yet a different FOG related cofactor within the developing heart.

Methods

All methods were performed by the author unless specifically stated otherwise.

Targeted mutagenesis of the murine GATA4 gene

An 8.2-kb Eco RV-Sac I fragment of murine GATA4 genomic DNA containing the N finger of GATA-4 (courtesy of Jeff Molkentin) was subcloned into pBluescript II KS (+/-) phagemid (Stratagene). By site-directed mutagenesis, Val 217 was changed to glycine (the codon GGC was changed to GTC; GeneEditor, Promega). (Site directed mutagenesis was performed with the help of J. Crispino). An intronic Bgl II site was changed to a Not I site to facilitate introduction of a floxed neomycin expression cassette. HSV-tk was cloned into a Sal I Site 5 of the homology region.

The targeting construct was linearized with Pvu I and electroporated into TL1 ES cells. (Technical instruction on the culturing protocol for ES cells was provided by Carole Browne). Electroporation was performed as follows: A 10 cm plate of ES cells was washed with 5 ml 0.05% trypsin/EDTA. 2.5 ml 0.25% trypsin/EDTA was added, and cells were incubated at 37° for 4'. An additional 2.5 ml of 0.25% trypsin/EDTA was added and suspension was pipetted to break up clumps. Cells were incubated at 37° C for an additional 2' and 5 ml complete medium (80% DMED, 15% FCS, 2% PEN/STREP, 1% nucleoside mix, 1% L-Glutamine, 1%NEAA, 10^-4 M 2ME, 10^3 u/ml LIF) was added. After pipetting, cell suspension was transferred to a 15 mL tube. Plates were washed with 5 mL medium and were added to the tube. Cell suspension was pelleted at 1000 rpm/8'/4°C. Cells were washed 2 x with HeBS, 1000 rpm/8'/4°C, and were resuspended at 1.3 x 10^7/ ml in HeBS. 25 ul (25-50 ug) of linearized targeting construct DNA was added to 0.75 ml of suspension for each electroporation. Cells were vortexed gently and incubated on ice for 10'. Cells were electroporated in 0.4 cm gap cuvette at 500 uF and 250V, and were then incubated on ice for 10'. Electroporated cells were plated in IEF (irradiated embryonic

fibroblast feeder cells) per protocol, in which electorporated cells were resuspended into 6 mLs of medium and divided into 6, 1mL suspensions added dropwise to each IEF plate. 24 hours following initial plating, G418 (280 mg/ml stock) was added for final concentration of 280 ug/ml. 4ul of gancyclovir (5 x 10^-3M stock) was added for a final concentration of 2 X 10^-6M.

Cells were fed daily with fresh G418, gancyclovir, and medium for 8 days. On days 7 and 8, clones were scored, picked, trypsinized, and transferred to IEF feeder plates using sterile technique. ES clones were allowed to grow until they reached 40-70% confluence, at which point they were frozen and expanded for growth of cells to be used for DNA extraction and analysis. 12-well gel-coated plates were filled with ES medium, and freezing vials were prepared each containing 150 ul freezing mixture (20% DMSO/80% FCS). Clones were trypsinized and dispersed into single cell suspension. Each well was rinsed with 0.5 ml 0.05% trypsin. 100ul 0.25% trypsin was added to each well, cells were incubated for 5' at 37°, 150 ul FCS was added to each well. 150 ul of this cell suspension was transferred to corresponding freezing vial, while the remaining cell suspension was added to corresponding well of DNA plate. Vials were placed in foam racks at -80°C before transferring to liquid nitrogen. DNA was isolated and purified from ES clones when cells were close to confluency. Each well was washed with 3 ml PBS. 600 ul lysis solution (GENTRA SYSTEMS) was added to each well, and cells were resuspended and pipetted into 1.5ml eppendorf tubes. Cells were incubated for 1 hour at 37 °C. 3 ul Rnase A solution (GENTRA SYSTEMS) was added to each eppendorf tube. Tubes were inverted 25X and incubated 1 hr at 37°. Protein precipitation was performed by cooling on ice 5 minutes, followed by the addition of 200 ul protein precipitation solution to each tube. Tubes were vortexed vigorously for 20 seconds, and centrifuged 14,000 rpm for 10 minutes at 4°C. Supernatant was poured into a new tube containing 600 ul isopropanol and was mixed by inverting 50x. Tubes were centrifuged at 14,000 rpm/3'/4°C. Supernatant was poured off,

and 600ul of 70% ethanol was added to each tube. Tubes were centrifuged again at 14,000 rpm/3'/4°C. Supernatant was poured off, and tubes were inverted and allowed to air dry. DNA hydration was performed using 100ul of DNA hydration solution (GENTRA) per pellet. DNA was heated 1 hour at 65°C and allowed to rehydrate overnight at RT, and was stored at 4°C.

Each sample of DNA was analyzed via Southern blot analysis in order to determine the presence of correctly targeted clones. The *Gata4* coding exon used as a probe used for Southern blot analysis is detailed in figure 1. This exon was prepared as a random-primed DNA probe using the Boehringer-Mannheim Random Primed DNA Labeling kit and was purified using Boehringer-Mannheim Quick Spin G-25 Sephadex columns. Southern blots were performed using standard technique. ES clones that were found to be correctly targeted (2 out of 100) were expanded for stock as follows. Positive clones were thawed to one well of an IEF plate and was fed with fresh medium after 24 hours. When wells were 50-70% confluent, they were split to 6 new wells of a 24 well IEF plate for P1 stock. When these wells were 50-70% confluent, 1 well was used for karyotyping, and 4 wells were frozen for stock. The GATA4 gene from two independently generated, targeted ES clones was amplified by PCR, sequenced, and found to be correctly mutated in one clone. Two clones were corectly integrated, however, only one clone had both neo and the point mutation. Sequencing was performed by sequencing facility at Children's Hospital and with the help of J. Crispino. This clone was injected into C57BL/6 blastocysts to generate chimeras. (Injection was performed by Y. Fujiwara).

Mating of mice and categorization of their offspring was done together in collaboration with A. Chapedelaine and J. Crispino. Genotyping was done thereafter by Southern blot analysis, as described in the legend of Figure 1C. (Both J. Crispino and M. Lodish contributed to genotyping) Tail and yolk sac DNA extraction was performed as follows: 500ul lysis buffer (100mM Tris HCL pH 8.0, 5mM EDTA, 0.2% SDS, 200mM NaCl) and 2.5 ul proteinase K solution (at 20 mg/ml) was added to eppendorf tubes

containing fresh cut or frozen tail samples. Tails were incubated overnight at 55° C. The following morning, tunes were vortexed and spun at 10 minutes, maximum speed. Supernatant was poured into new tube containing 500 ul isoporpanol and shaken well. Tubes were spun an additional 2 minutes, maximum speed, and supernatant was poured off. 1mL 70% ETOH was added to each tube which was then briefly vortexed. Samples were spun down, washed with ETOH, air-dried, and resuspended in 50 ul of 0.1 M Tris-HCL pH 8.0, 0.1 M EDTA. Samples were analyzed via Southern blot as per standard protocol.

Histological analysis

Mouse embryos were isolated from GATA4 heterozygous knock-in matings between E10.5 and 13.5. Embryos were fixed in Bouin's, dehydrated into increasing concentrations of ethanol, transferred into xylene, and sectioned in paraffin at 6 μm and stained with hematoxylin and eosin. Sectioning was performed independently by both the author and J. Crispino. Our slides were shared with Silvio Litovsky, a cardiac pathologist at Brigham and Women's hospital.

Immunohistochemistry

Murine tissue used for immunohistochemistry was fixed in 10% formalin overnight, processed, and paraffin embedded using standard histologic techniques. Tissue sections 4μ thick were dewaxed in xylene then re-hydrated by passage through graded alcohol solutions. Sections were immersed in 10 mmole/L citrate buffer (pH 6.0) in a thermoresistant container and heated in a microwave oven (800 W,General Electric) at 199°F for 30 min. A rabbit polyclonal antibody to GATA-4 (sc-9053, Santa Cruz Biotechnology) was used at a 1:50 dilution and detected by the Rabbit DAKO Envision Plus System, Peroxidase DAB (Dako). Negative controls were performed by substituting the primary antibody with species-and isotype-matched, nonimmune immunoglobulins. Additional controls included omission of the primary antibody as well as substitution of the

primary antibody of interest with one of differing specificity. (Immunohistochemistry of embryo sections was performed by Beth Thurberg, Cardiac pathologist at Brigham and Women's Hospital, while genotyping of embryos and embryo preparation for sectioning was performed by the author).

Whole-mount staining of hearts with the αFlk-1 and α ICAM-2 antibodies (obtained from Pharmingen) was performed independently by the author as well as by J. Crispino as follows. Embryos were collected in PBS and hearts were dissected out. Hearts were fixed in methanol: DMSO (4:1) overnight at 4°C. Hearts were transferred into methanol:DMSO:30% H_2O_2 (4:1:1) for 4-5 hours at RT. Hearts were rehydrated through decreasing concentrations of methanol into PBS. Hearts were incubated in PBMST (3% instant skim milk powder, 0.1% Triton X-100 in PBS) for 1 hour at RT, followed by overnight incubation with primary antibody diluted in PBSMT (1:50). The following morning, hearts were washed in PBMST twice at 4°C and 3 times at RT for 1 hour each. Hearts were incubated overnight at 4°C with the secondary antibody in PBMST at 4°C overnight. HRP coupled goat anti-rat IgG was used at a 1/100 dilution. The following morning, 5 washes in PBMST for 1 hour each were completed. Hearts were incubated in developing solution (sigma) for 20 minutes at RT. H_2O_2 was then added to 0.03% final concentration. Hearts were rinsed in PBT (0.2% BSA, 0.1% Triton X-100 in PBS) and then PBS for 5 minutes x 2 at RT, and then were fixed in 2% paraformaldehyde/ 0.1% glutarldehyde/ PBS at 4°C overnight.

In situ hybridization analysis

Whole mount hybridization was performed using riboprobes labeled with digoxigenin-UTP. Animal hearts were isolated surgically from embryos through dissection in PBS. Hearts were then fixed in 4% paraformaldehyde at 4° C overnight with gentle rocking. Hearts were washed twice in PBT for 5 minutes at 4° C and were dehydrated into increasing concentrations of methanol at room temperature. Digoxigenin-

UTP labeled RNA probe was synthesized as follows: the following reagents were mixed at room temperature, 10µl sterile distilled water, 4µl 5 x transcription buffer, 1µl 0.2M DTT, 2µl nucleotide mix, 1µl linearized plasmid (1µg/µl), 1µl placental ribonuclease inhibitor, 1µl RNA polymerase. The reagent mixture was incubated at 37° for 2 hrs. 2µls of Dnase I were added, along with 100µls of TE, 10µls 4M LiCl, and 300µl ethanol. Mixture was incubated at -20°C for 30 minutes, spun in a microcentrifuge at 4°C for 10 minutes, washed with 70 ethanol and air dried. The pellet was then re-dissolved in TE at 0.1µg/µl and stored at -20°C.

Pretreatment and hybridization of hearts was carried out as follows: Samples were rehydrated through decreasing concentrations of methanol in PBT, then bleached in 6% hydrogen peroxide in PBT for 1 hour at RT. Samples were then washed with PBT three times for 5 minutes each, then treated with 10µg/mg proteinase K in PBT for 15 minutes at RT. Embryos were then washed with 2 mg/ml glycine in PBT, followed by PBT for 5 min at RT. Embryos were re-fixed with 0.2% glutaraldehyde/ 4% paraformaldehyde in PBT for 20 minutes at RT, followed by additional washes in PBT. Embryos were incubated in prehybridization solution (50% formamide, 5X SSC pH 4.5, 50µg/ml yeast RNA, 1% SDS, 50µl/ml heparin) for 1 hour at 70°C. Prehybridization solution containing 10µ probe was added, mixed, removed, and sample was incubated overnight at 70°C in 1 ml of hybridization solution. Post-hybridization washes were performed and embryos were preblocked with 10% sheep serum in TBST at RT for 2.5 hours. Blocking seurm was removed, 200 µl antibody solution was added to the embryos, which were then rocked overnight at 4°C. The following morning, hearts were washed in TBST 3 times for 5 minutes each, 5 times for 1 hour each, and finally overnight. The following morning, hearts were washed 3 times with freshly prepared NTMT for 10 minutes each at RT. Hearts were incubated with developing solution (Boeringer Manneheim) in the dark for 4 hours. When reaction had completed, embryos were washed 2X with NTMT for 10 min at RT, reaction was stopped with PBT pH 5.5 for 10 minutes RT, hearts were post-fixed in 4%

paraformaldehyde in PBT + 0.1% glutarldehyde, and washed in increasing concentrations of glycerol to stabilize hearts for photography.

Antibody solution was prepared as follows: 0.5 ml TBST was added to 3mg embryo powder and allowed to incubate at 70°C for 30 minutes. Sample was vortexed for 10 minutes, then cooled on ice. 5µl sheep serum and 1µl of anti-dig AP (Boeringer Manneheim) were added. Sample was shaken gently at 4°C for 1 hour, then spun for 10 minutes at 4°C, and finally the supernatant was diluted to 2 ml with 1% sheep serum in TBST.

Results

We sought to establish an intrinsic role for GATA-4 in heart development by generating mice harboring a knock-in mutation that cripples its interaction with FOG-2 or other FOG-factors. Residue 217 of GATA-4, which corresponds to Val 205 of GATA-1, was changed to glycine by gene targeting in embryonic stem (ES) cells. This residue faces away from DNA (Fig.1A) and lies within the GATA –N finger:FOG interface. Substitution with glycine disrupts interaction with either FOG-1 or FOG-2 and leaves DNA-binding properties of GATA factors unperturbed. Targeted mutation of murine *Gata4* was accomplished with the construct depicted in Figure1B. A floxed neomycin resistance cassette was incorporated into an intron downstream of the exon containing V217. ES cells harboring both the V217G mutation and the floxed neomycin cassette were injected into host blastocysts to generate chimeras. *Gata4* +/ki mice appeared normal. No liveborn homozygotes resulted from interbreeding of heterozygous offspring, indicating the *Gata4* ki/ki embryos are embryonic lethal. Southern blotting of timed matings (Fig.1C) demonstrated that *Gata4* ki/ki embryos die between E11.5 and E13.5. Their gross appearance was remarkably similar to that of *Fog2* -/- embryos (20, 21). *Gata4* ki/ki embryos are pale and edematous, compared with wild-type littermates, and peripheral hemorrhage is often observed (Fig.2A,B).The neomycin cassette was removed in some strains by interbreeding with a Cre-recombinase-deleter strain (22).No differences in phenotype were observed between strains containing or lacking the neomycin cassette. Furthermore, heterozygotes were born at the expected frequency and displayed no detectable phenotype, arguing that the GATA-4 mutant protein does not act in a dominant negative manner.

Wild-type and the GATA-4 ki/ki embryonic hearts at E12.5 were examined in serial sections, cut in the transverse plane, and inspected from the cephalic to the caudal aspect of the specimens (Fig.2C –H).*Gata4* ki/ki hearts revealed a double-outlet right ventricle where both great arteries arise from the right ventricle (Fig.2F).The ventricular septal defect is the

only outlet for the left ventricle (Fig.2F). In addition, the endocardial cushion cells of both the pulmonary and aortic outflow tracts appear more numerous than in wild-type embryos. Furthermore, robust mitotic activity is evident in these areas, and the pulmonary and aortic outflow tracts comingle in some planes of section. In wild-type embryos, however, the pulmonary and aortic outflow tracts display distinct endocardial cushions (Fig. 2E). Right and left atria of the mutant are massively dilated and freely communicate (Fig.2D,H),and a common atrioventricular valve opens into the center of a large ventricular cavity, which lacks a ventricular septum (Fig.2H). In contrast to a wild-type heart, in which two distinct atrioventricular valves (the tricuspid and mitral valves;Fig.2G) develop in continuity with the septum and with the aortic valve, respectively (Fig.2E), the common AV valve in the mutant heart has no fibrous continuity with the aortic valve. In addition, *Gata4* ki/ki myocardium appears thin (cf.Fig. 2G,H).These abnormalities were consistently seen in the GATA-4 ki/ki embryos. Similarly, complete penetrance was also observed in FOG-2 -/- embryos (21).

The morphology of *Gata4* ki/ki hearts resembles that seen in *Fog2* -/- embryos, however a few key differences exist. Both the *Gata4* ki/ki and *Fog2* -/- hearts exhibit enlarged atria, thin myocardium, ventricular septal defect, and a common AV valve. However, while the *Fog2* -/- hearts have a severely obstructed pulmonary trunk, *Gata4* ki/ki hearts were unique in terms of the presence of a double-outlet right ventricle. In addition, *Gata4* ki/ki hearts had increased cellularity in the semilunar valves and outflow tracts that was not observed in the *Fog2* -/- hearts.

To validate that the observed phenotype results from expression of a qualitatively different GATA-4 protein rather than from an altered pattern, or level, of expression, we examined GATA-4 protein expression. Wild-type and *Gata4* ki/ki E12.5 hearts were sectioned in the sagittal plane and immunostained for expression of GATA-4 (Fig.3A,B).At this stage, GATA-4 protein is present in both endocardium and myocardium of developing atria and ventricles. Immunostaining is strongest in surface endothelial cells and mesenchymal cells of the endocardial cushion tissues, which develop into the semilunar

(Figs.3B,F)and atrioventricular valves (Fig. 3D,H). GATA-4 protein is also present in the embryonic liver and gut epithelium in both wild-type and mutant embryos, as expected (Fig.3A,E; 23). Taken together, these studies demonstrate that GATA-4 ki protein is expressed comparably to wild-type protein.

In several respects, the morphology of *Gata4* ki/ki hearts resembles that seen of *Fog2* -/- embryos. A distinctive feature of *Fog2* -/- hearts is the absence of coronary vasculature (21).To examine the status of their coronary vasculature, *Gata4* ki/ki hearts were immunostained for two endothelial cell markers. Flk-1, the receptor for vascular endothelial growth factor (VEGF), is essential for vascular development throughout the mouse (24). Flk-1 is not detectable in *Gata4* ki/ki hearts, though lung tissue stains intensely (Fig.4A). Similarly, abundance of the intracellular adhesion molecule-2 (ICAM-2) is reduced in *Gata4* ki/ki hearts; note the absence of the developing capillary plexus (Fig.4B, arrows). These findings are indistinguishable from those reported previously for *Fog2* -/- hearts, which exhibited an identical early block in the formation of coronary vessels (21). As such, they provide strong evidence that FOG-2 serves as a transcriptional cofactor for GATA-4 in myocardium as an essential step in the initiation of coronary vascular development.

Normal vessel development occurs as epicardial cells undergo an epithelial-to-mesenchymal transition, giving rise to the subepicardial coronary vasculature (25). Studies of *Fog2* -/- embryos showed that despite formation of an intact epicardial layer and expression of epicardium-specific genes in *Fog2* -/- hearts, markers of cardiac vessel develpoment (ICAM-2 and FLK-1) were not detected. Our current hypothesis is that the interaction between GATA-4 and FOG proteins is essential for the development of coronary vasculature from epicardium. We postulate that this specific protein-protein interaction is nescessary for the critical step of epithelial to mesenchymal transformation, as represented by the failure to activate FLK-1 and ICAM-2 expression (Figure 5).

Of numerous myocardial-expressed genes assayed in *Fog2* -/- hearts, only the basic –helix –loop helix eHand and dHand genes were altered in their expression on loss of *Fog2*

(21). To assess this phenotype in *Gata4* ki/ki hearts, the expression of Hand genes was examined by RNA in situ hybridization. Expression of eHand is reduced in the myocardium of *Gata4* ki/ki hearts relative to that seen in wild-type hearts (Fig.4C). Interestingly, whereas the expression of eHand in the outer curvature of the ventricles is reduced (Fig.4C, white arrows), its expression in the outflow tract is increased (Fig.4C, dark arrows).This increased staining likely results from the increased cellularity within this region of the *Gata-4* mutant hearts and was not observed in *Fog2* - deficient hearts (Fig.2F).In contrast, the expression of dHand is only marginally reduced in the mutant heart (Fig 4D).

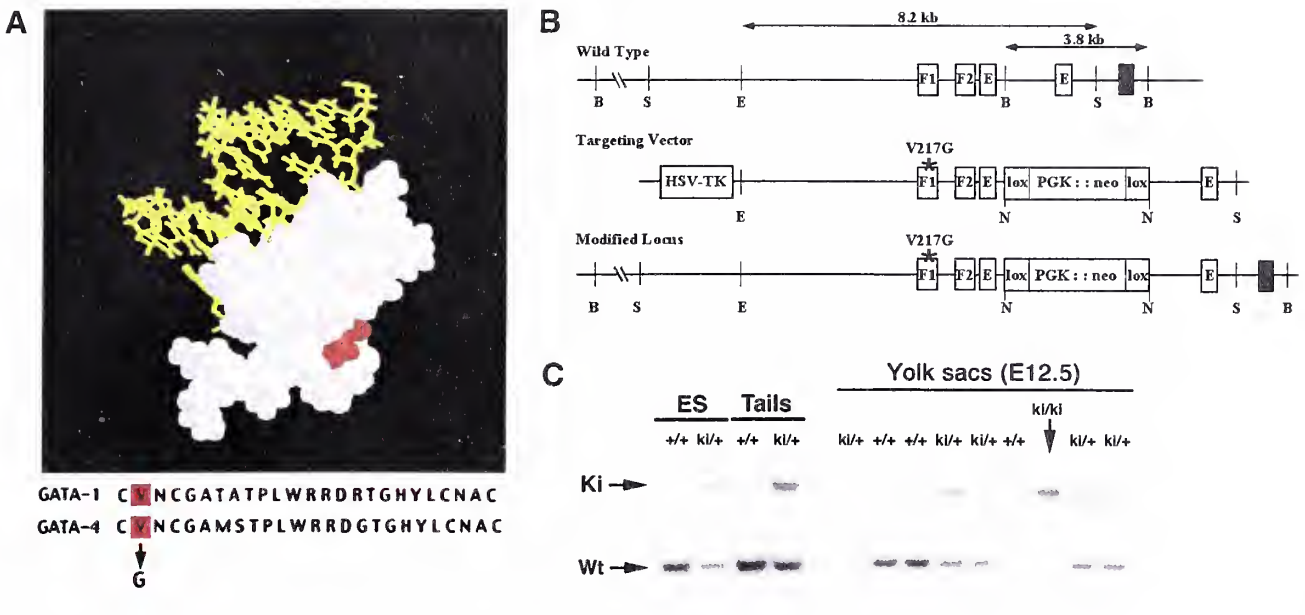


Figure 1. Targeting the GATA4 –FOG-2 interaction in mice.(A)Structure of the N finger of chicken GATA-1 modeled with DNA. The essential valine is highlighted in red in both the illustration and in the sequence alignment of the murine factors, GATA-1 (V205) and GATA-4 (V217),shown below. Note the high conservation of the residues within the N finger of the two GATA proteins.(B) Partial restriction map of the murine *Gata4* wild-type locus (top), the *Gata4* knock-in targeting vector (middle),and targeted homologous recombination before excision of the selection cassette (bottom).The targeting construct contains the HSV-tk and neomycin resistance (neo R) genes under the control of the mouse phosphoglycerate kinase (PGK) promoter. Homologous recombination results in replacement of wild-type *Gata4* with genomic DNA harboring a substitution of valine to glycine at position 217 in the N finger of GATA-4, as well as the incorporation of neomycin cassette. *Gata4* coding exons are shown as empty boxes, whereas the exon used as a probe used for Southern blot analysis is highlighted by a black box. S, Sac I;E,Eco RV;B,Bgl II;N,Not I.(C) Southern blot analysis of ES cell DNA and mouse tail DNA (left panel)showing the presence of heterozygous mutant animals (ki/+). Analysis of E12.5

embryos resulting from an intercross of *Gata4* knock-in heterozygotes (ki/+), demonstrating the presence of all expected genotypes (right panel).The wild-type allele (WT) generated a 3.8-kb band after digestion of genomic DNA with Bgl II. In contrast, the knock-in mutated allele (Ki) generated a much larger fragment because of the replacement of the intronic Bgl II site with Not I.

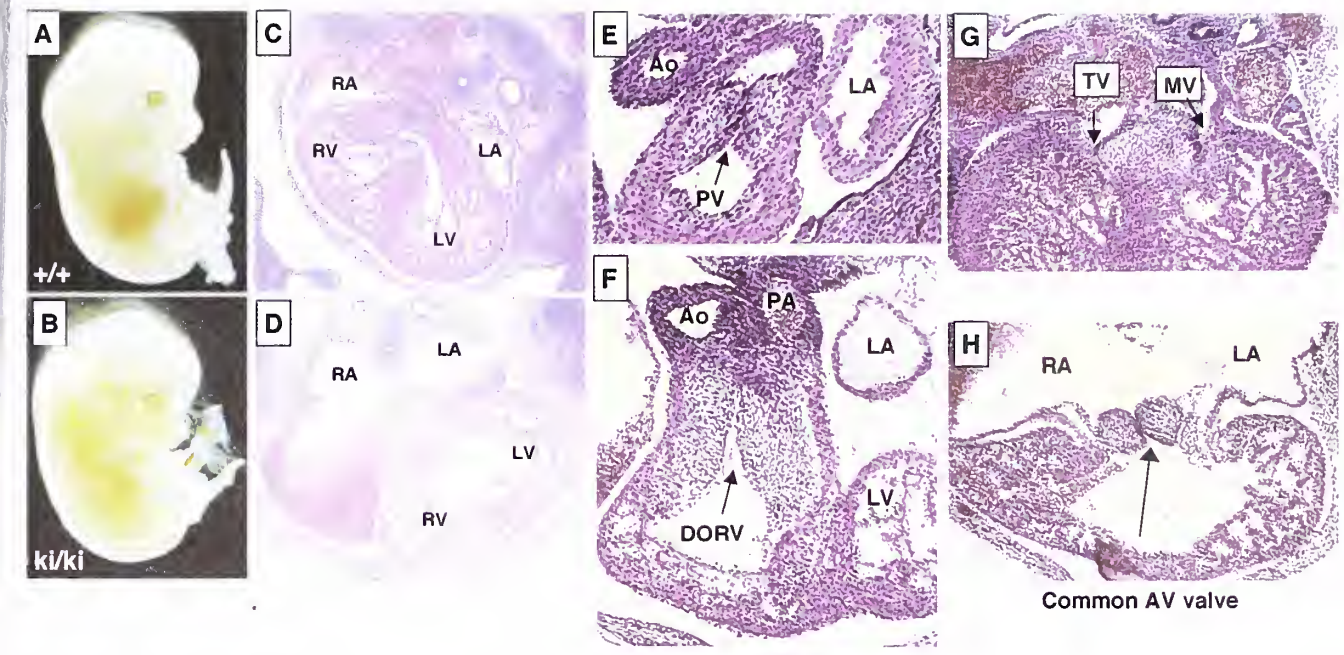


Figure 2. Heart defects in *Gata4* mutant (ki/ki)embryos. (A ,B) 3 homozygote, complete mutant embryos from separare litters were compared with 3 corresponding wild-type littermates. Wild-type (A)and mutant (B) embryos at E13.5 showing edema and peripheral hemorrhaging in a mutant.(C ,D)Transverse sections through wild-type (C)and mutant (D) hearts at E13.5 at the level of the atrioventricular (AV) junction show enlarged atria, thin myocardium, and the absence of a ventricular septum. Original magnification, 40 x.(E ,F) Transverse sections of wild-type (E) and mutant (F)hearts at the level of the aortic and pulmonary outflow tracts. *Gata4* ki/ki hearts have a double outlet right ventricle, in which all blood exits the heart into both great arteries, the pulmonary artery and the aorta. The left ventricle, which normally delivers blood to the aorta, fails to communicate with an artery in the mutant. Also note the apparent increase in cellularity of both outflow tracts and semilunar valves in the mutant. Original magnification, 400 x.(G ,H) Transverse sections of wild-type (G)and mutant (H)hearts at the level of the AV junction. *Gata4* ki/ki hearts form a common AV valve that is situated between the left and right ventricles. For comparison, the mitral (MV) and tricuspid (TV) valves of the wild-type heart are indicated by arrowheads. Original magnification, 100x.

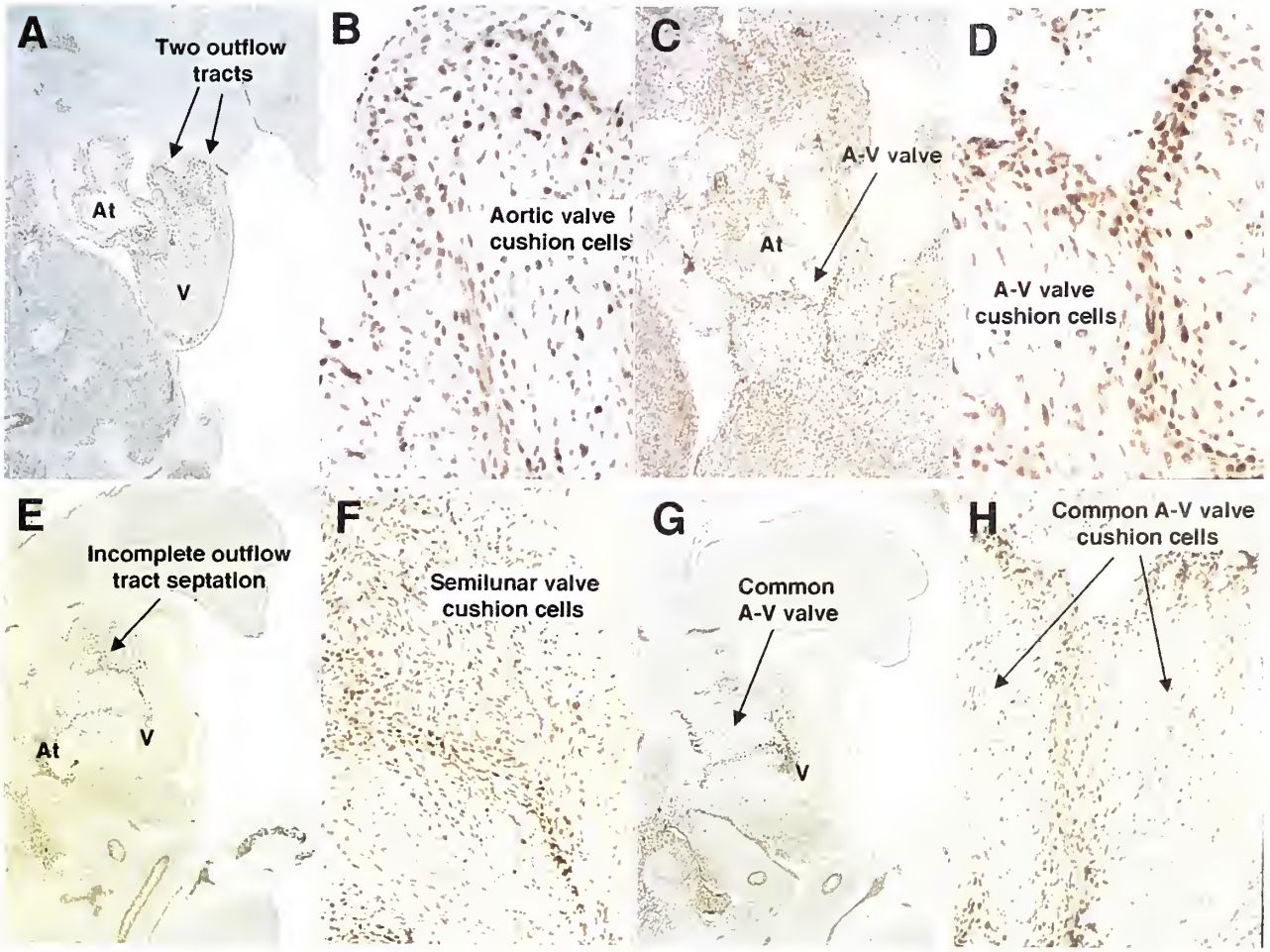
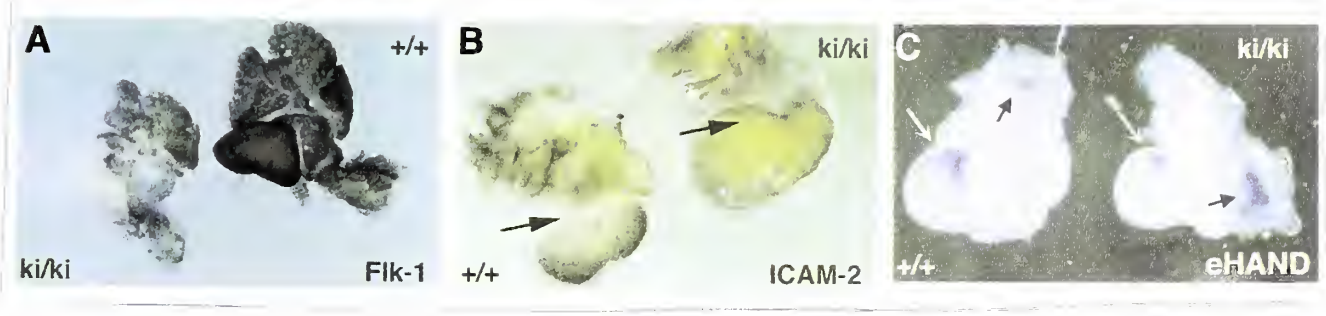


Figure 3. Expression of *Gata4* in the heart. Sagittal sections of wild-type (A –D)and *Gata4* ki/ki (E –H)embryos at E12.5 were stained with an ∝GATA-4 antibody. Both wild-type and mutant hearts display similar staining within the semilunar and AV valve cells. Note the staining of outflow tracts in both the wild-type and the mutant heart. At, atrium; AV, atrioventricular; V, ventricle. Original magnification, A,E,G,40x; B,D,F,H,400x; C,100x.



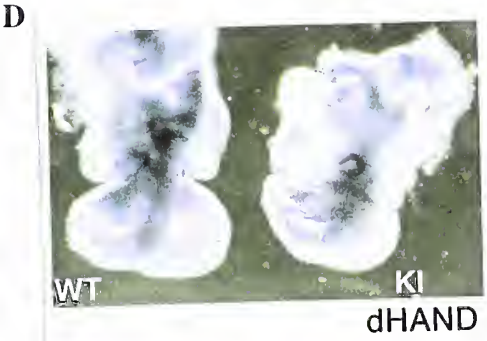


Figure 4. Aberrant expression of coronary vessel and myocardial transcripts.(A) Staining of E12.5 wild-type (+/+) and mutant (ki/ki) hearts with an αFlk-1 antibody (dorsal view). N = 2. Staining of the mutant is vastly reduced within the heart, but lung tissue stained with equal intensity.(B)Immunostaining of wild-type and mutant hearts at E12.5 using the αICAM-2 antibody (dorsal view). N =2. Note the absence of a well-developed vascular tree in the mutant heart.(C)Whole-mount RNA in situ staining of eHand in E11.5 hearts. N=3. eHand expression is down-regulated in the outer myocardial layer (white arrows),whereas there is more intense staining in the outflow tract of the mutant (dark arrows).Note that the direction of the outflow tract relative to the heart is altered in the mutant, consistent with the pathological findings. (D) Whole-mount RNA in situ staining of dHAND in E 11.5 hearts. N=3. The expression of dHand (blue) is only marginally reduced in the mutant heart.

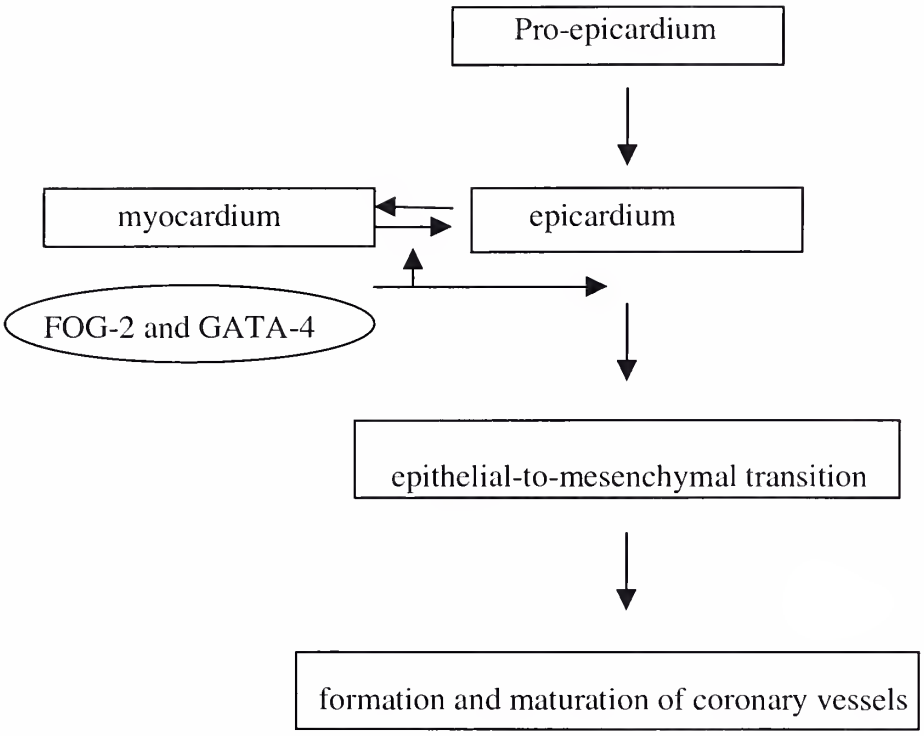


Figure 5. Model of arrest in development of coronary vasculature caused by disruption of GATA-4 and FOG-2 proteins. FOG-2 serves as a transcriptional cofactor for GATA-4 in myocardium at an essential step in the initiation of coronary vascular development, the initiation of epithelial to mesenchymal transformation of epicardial cells. (Modeled after Figure 7, reference 21).

Discussion

Our data point to essential roles for the GATA-4:FOG-2 interaction in heart morphogenesis and coronary vascular development. The power of our analysis rests on the exquisite specificity of the knock-in mutation within the N finger of GATA-4. The residue we have chosen to modify is required for physical interaction with FOG-like proteins and does not influence the DNA-binding specificity of the GATA-protein (6). Although displaying many similar features, *Gata4* ki/ki hearts are distinguished from *Fog2* -/- hearts, however, by the presence of a double-outlet right ventricle and defects in the semilunar valves and outflow tracts. As immunostaining confirmed that GATA-4 is expressed at wild-type levels in the semilunar valves of the *Gata4* mutant heart, it is likely that another FOG, or FOG-like protein, that functions as a cofactor for GATA-4 in transcription is expressed in these valve cells. Though high-level expression of the only other known vertebrate FOG-like factor, FOG-1, has not previously been observed by in situ RNA hybridization, FOG-1 transcripts are present at low levels in Northern blots of total heart RNA (A.P.Tsang and S.H.Orkin, unpubl.). Thus, it is quite possible that disruption of the physical interaction between GATA-4 and FOG-1, or a novel, undefined FOG protein, is responsible for impaired development of the semilunar valves and the appearance of a double-outlet right ventricle.

Given the profound effects of mutation of either GATA-4 or FOG-2 proteins on heart morphogenesis in mice, it is worth considering their potential relevance to human congenital heart defects, such as the Tetralogy of Fallot or the double-outlet right ventricle (26). It is therefore logical to evaluate FOG and GATA factors as candidate genes for congenital cardiac defects in humans. Indeed, haploinsufficiency at the GATA-4 locus is often seen in patients with del(8)(p23.1) and congenital heart disease, and it has been postulated that this GATA-4 deficiency may contribute to the phenotype of patients with monosomy of 8p23.1 (27). Further elucidation of the complex pathways of transcription

factors modelling cardiogenesis will ultimately help us to better understand cardiac development and congenital heart disease.

Whereas mutation of several genes, such as Jmj (Jumanji), Sox4 ,and Egfr /Shp2, give rise to the double-outlet right ventricle defect in mice (28, 29, 30), and defects in other genes for transcription factors, such as FOG2, NF1, neurotrophin 3, and RXR ∝ result in all or a subset of the Tetralogy of Fallot (31, 32, 33, 34, 21), the consistent and combined phenotype seen in the *Gata4* ki/ki mice is unique. Although it is sometimes difficult to distinguish between double-outlet right ventricle with associated pulmonary stenosis and the Tetralogy of Fallot, it is clear that the defects in the GATA-4 ki/ki hearts are different in the outflow tracts than those observed in *Fog2* -deficient embryos.

Through the use of an altered specificity mutant, we demonstrate that GATA-4 very likely requires both FOG-2 and an additional FOG, or FOG-like protein, as cofactors for distinct aspects of heart development. Interaction with FOG-2 is essential for the initiation of coronary vasculature and for some morphogenetic events, whereas interaction with a distinct FOG protein appears to be required for formation of cardiac valves. Our results are surprising in that two other GATA-factors, GATA-5 and GATA-6, are also expressed in myocardium, and indirect data have suggested that they might compensate for the absence of GATA-4. For example, previous studies show that GATA-4 is dispensable for terminal differentiation of cardiomyocytes and that *Gata4* -/-ES cells contribute to all layers of the heart. In these experiments, it has been suggested that GATA-5 or GATA-6 functionally replace GATA-4 (35). It is possible that proper expression of the GATA-4 ki/ki protein, as distinguished from the absence of GATA-4 in the knock-out situation, precludes compensation by other GATA factors. Indeed, immunostaining with an ∝ GATA-6 antibody demonstrated that GATA-6 expression, though similar to that of GATA-4, is not up-regulated in the *Gata4* ki/ki hearts (data not shown). In addition, staining with ∝GATA-5 antibody revealed a normal pattern in *Gata4* ki/ki hearts (data not shown). As GATA-5 is

no longer expressed within the ventricles of the heart at E12.5, it is unlikely that it would compensate for the absence of functional GATA-4 (8).

Our findings implicate GATA-4 as the principal GATA factor relevant to heart morphogenesis and coronary vasculature development and as the primary partner for FOG proteins in the heart. This represents the second example of transcriptional regulation involving GATA –FOG protein complexes and argues for their broad involvement as key regulators of multiple developmental pathways.

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